*GENE CONTROL OF MAMMALIAN PIGMENTARY DIFFERENTIATION, I. CLONAL ORIGIN OF MELANOCYTES**

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In higher organisms, the cells of an individual become greatly diversified despite their identity of genotype. How such diversification is achieved and how supracellular organization then comes about have remained largely obscure. A new way of getting at these questions was formulated in this laboratory; its purpose was to subject the pivotal genotype-phenotype relationship to experimental manipulation. The intact organism was taken to be the necessary framework for such an experimental study of gene expression, and the mouse, with its wide variety of available genetic markers, was easily the most promising vertebrate species. The plan was to make artificial mice: within each, cells with *different*, rather than identical, genotypes would be included.[1]

Certain kinds of mosaicism had previously been extensively employed in studies with *Drosophila*.[2] The first indication, in a mammal, of an admixture of genotypes came with Owen's discovery of erythrocyte mosaicism in fraternal cattle co-twins.[3]

Efforts to devise suitable techniques were begun in 1960 and eventually met with success (Fig. 1). Procedural details have been presented elsewhere.[1] Developing eggs are removed from donor females within the period from two cells through morula, and are explanted to a serum-containing medium. The surrounding membrane (zona pellucida) is digested away with 0.5 per cent pronase, and rinsed, denuded eggs of similar stage and different genotypes (shown as black and white) are placed in contact at 37°C for adhesion between apposed blastomeres. During subsequent culture, cells migrate randomly within each aggregate, forming a composite sphere which becomes a single blastocyst (after 1 day, if explants were at the 8-cell stage). Blastocysts are then surgically transferred to the uterus of an "incubator" mother (usually of the ICR strain), who is pseudopregnant following mating with a tested, sterile (vasectomized) male on the day after the fertile matings of the egg donors. Parturition occurs at the normal time, and surrogate mothers generally take good care of the young. Size-control mechanisms restore normal embryo size shortly after implantation, thus obviating any necessity for employing half-embryos rather than entire embryos in the original aggregated pair. Striking though this regulation may seem, it is not surprising if viewed as an exaggerated manifestation of the normal mechanisms which must exist to restrict growth of organized embryos in contrast to unrestrained proliferation of embryo cells in cell culture.

The first viable, demonstrably mosaic mice of quadriparental lineage were born in 1965.[1] Since then, some 500 morphologically normal adult animals have been produced by these methods, and many have by now lived out a full lifespan. They, in turn, have left over 25,000 offspring.

As would be expected from the early stage at which the cells are admixed, all tissues in the mosaics can consist of two genotypes throughout the life of the animal. In large numbers of individuals, cells co-exist with different alleles at the

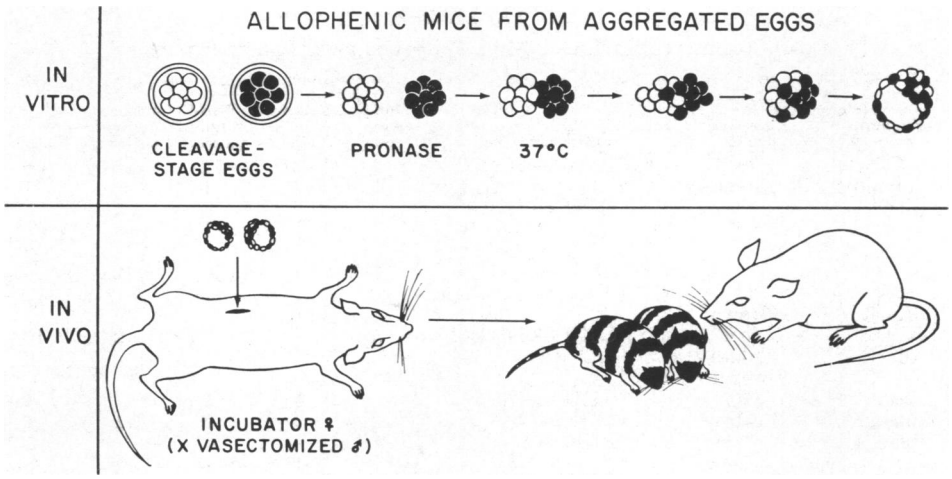


FIG. 1.—Diagram of the experimental procedures for producing allophenic mice.

strong *histocompatibility-2* (*H-2*)-locus, governing graft acceptance. Both types of antigens are produced, with no adverse effects whatever, and a permanent state of immunological tolerance is found.[4, 5] In addition, cells of *lethal* genotype (e.g., *WW*) can be made to survive in the company of nonlethals (Table 1). Another of the original experimental aims, that of removing all restrictions on choice of component genotypes, has therefore also been realized.

Numerous combinations of genetic markers have been incorporated into mosaics. They have provided new insights into basic aspects not only of differentiation, but also of disease, aging, and behavior.

The present paper begins a series of reports on gene control of pigmentary differentiation. The melanocytes of the coat are a favorable experimental starting point because their distribution is readily observed; markers influencing them are known at many loci; and naturally occurring color patterns are common, for comparison with patterns in artificial color mosaics. The existence of natural patterns has itself often been cited as an illustration of the dilemma in differentiation, since a single genome is producing quite distinctive subpopulations of pigment cells which are arranged in a geometry peculiar to the genotype.

The projected pigmentary series will cover many topics; for purposes of orientation, a brief survey follows: (*a*) Analyses of the mosaics have disclosed that all melanocytes in their coats are clonally derived from a fixed, small number of primordial melanoblasts. The same clonal plan also holds for ordinary (nonexperimental) mice. (*b*) Genes which determine that these cells will be melanoblasts first become active in them at a definable time in early embryonic life, prior to any visible evidence of neural crest. (*c*) Gene activity remains stabilized through many successive cell generations, during which proliferation occurs in a similar manner in all clones, unless selection occurs. (*d*) Many (possibly most) genotypes can cause the *primordial* melanoblast population in an individual to consist of two or more different cellular phenotypes, as a result of several possible kinds of genetic control mechanisms. (*e*) When such diverse cells co-exist, their primary distribution is not

TABLE 1

COAT COLORS IN ALLOPHENIC MICE PRODUCED FROM PAIRED EMBRYOS

Melanocytes: Dominant color	↔	Melanocytes: Recessive color	Strains combined	Single-Color Mice: Dominant color	Single-Color Mice: Recessive color	Two-Color Mice: Standard pattern	Two-Color Mice: Derived patterns	Totals
CC	↔	*cc*	C57BL/6 ↔ ICR	1	9	11	5	26
Colored		Albino	C57BL/6 ↔ BALB/c	3	5	1	2	11
(black)			C3Hf ↔ ICR		5	2	1	8
			C3Hf ↔ BALB/c	2	2	1		5
			C3Hf ↔ AKR	8				8
			C3H/DiSn ↔ C3H.K	7	1	6	2	16
BB	↔	*bb*	C57BL/6J ↔ C57BL/6J-*bb*			1		1
Black		Brown						
BBDD	↔	*bbdd*	C3Hf ↔ DBA/2	5	2	1	2	10
Black		Dilute brown						
LnLn	↔	*lnln*	C3H ↔ fzln		4	1		5
Intense		Leaden	C3Hf ↔ fzln	2		2	1	5
(black)			C57BL/6 ↔ fzln	1				1
			cr ↔ fzln	1	1	1		3
MiMi	↔	$mi^{bw}mi^{bw}$	C3Hf ↔ C57BL/6-$mi^{bw}mi^{bw}$	1	3			4
Pigmented (black)		White						
WWBBDD	↔	*wwbbdd*	*WH-Ww* ↔ DBA/2	2			1	3
White		Dilute brown						
W^vW^vBBDD	↔	*wwbbdd*	C57BL/6J-W^vw ↔ DBA/2	2	1			3
White		Dilute brown						
			Grand Totals	35	33	27	14	109

The symbol "↔" designates the genotypes or strains combined into one individual by aggregation of eggs. Only *genetic differences directly affecting melanocytes* are shown in the first column, with coat color for each genotype; colors in parentheses are due to other genes which are identical in both pigmented members, or detectable only in the pigmented partner of an albino. The fzln and cr inbred strains are local ones which also carry mutations affecting hair structure (*fz* and *cr*, respectively). *Cc*, *Bb*, *BbDd*, and *Lnln* adult mice, heterozygous for alleles listed in the first four genotypic combinations, are single-color; the color patterns that normally occur in heterozygotes with certain alleles at the *Mi*- and *W*-loci will be reviewed elsewhere.

random with respect to each other but follows a given arrangement so that, after cloning is complete, a reproducible color pattern is found. Alternative products of only one pigmentary locus appear sufficient to mediate cell recognitions and to dictate preferential associations among early melanoblasts. (*f*) Selection plays a major role in normal development of all tissues and systematically leads to gene-specific modifications of clonal histories and, therefore, of the final total, or organismic, phenotype (e.g., color pattern). (*g*) Mammalian pigmentary "genes" appear to be highly complex entities. Apart from their direct melanizing functions in melanoblasts, many (perhaps all) have other expressions in developing hair follicles. (*h*) Indirect or inductive effects influence hair follicle development. (*i*) After melanoblasts enter follicles, further alterations of pigment cell phenotype and of color pattern are produced.

The clonal origin of melanocytes will be described below.

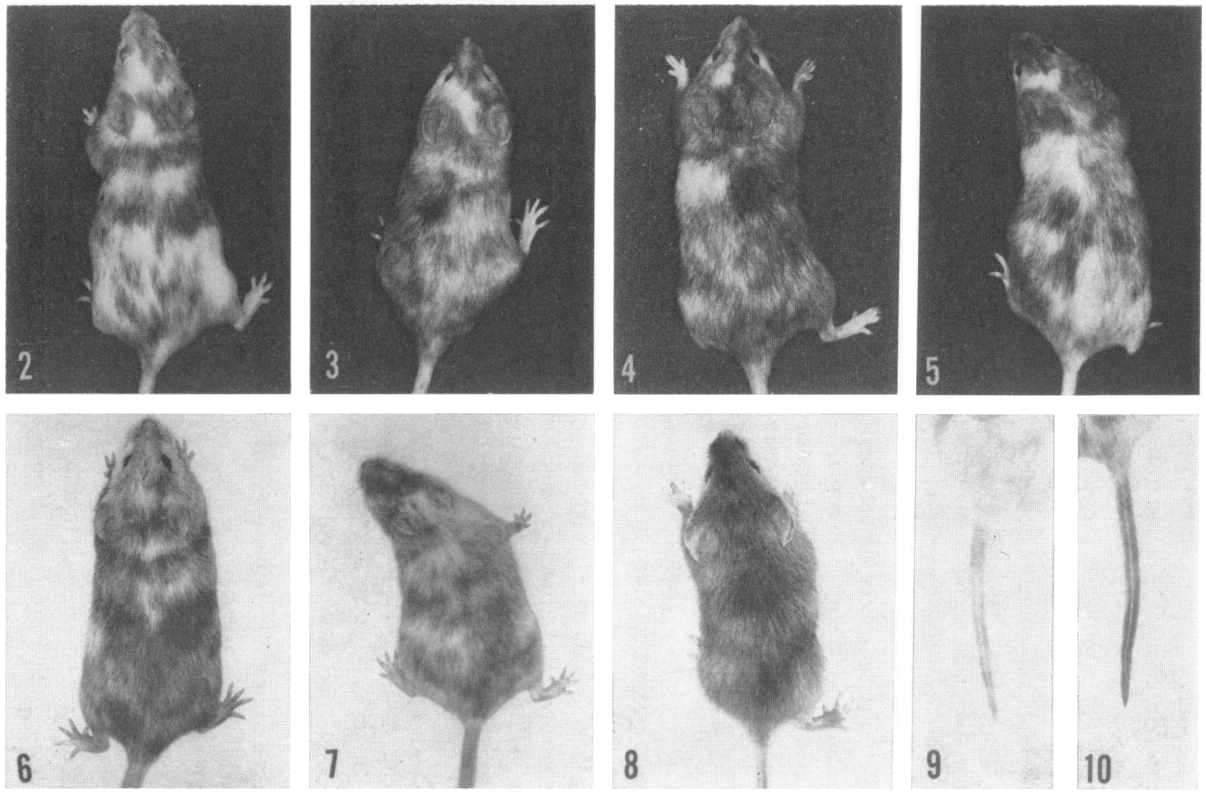
Materials and Methods.—Component genotypes: Seven combinations of *homozygous* melanocyte genotypes employed are listed in Table 1. The *C*-allele is required for presence of pigment, with actual color depending upon alleles at other loci (in these *CC* cases, black); *cc* is *albino*. Silvers' work[6] has established that melanocytes are present in hair follicles of *cc*, though they remain colorless or "amelanotic"[7] due to lack of tyrosinase.[8] Thus a *CC* ↔ *cc* genetic mosaic contains real pigmentary cells in the white areas. Other white or white-spotted genotypes,

on the other hand (e.g., WW, W^vW^v, $mi^{bw}mi^{bw}$), do not have detectable cells of melanocyte type in their white hairs.[7, 9] The *B*-allele produces *black* pigment; *bb* is *brown*. Both *dilute* (*dd*) and *leaden* (*lnln*) genotypes cause clumping of pigment granules and reduction in dendritic extensions, and lighten any color appreciably, while their respective *D*- and *Ln*-alleles produce full color.[10] It should be emphasized that secondary pattern effects are indirectly caused by the *AA* (*agouti*) $\leftrightarrow$ *aa* (*nonagouti*) differential in some animals and that these results will be discussed elsewhere; only primary activities in the melanoblasts themselves will be considered here. The largest group of experiments involved black (*CC*) and white (*cc*) composites because these genotypes were most readily available on a variety of strain backgrounds, affording an opportunity to observe any significant influences of the residual genotypes. Comparison of results in all $CC \leftrightarrow cc$ individuals was then made with the other series in which both genotypes in each pair were pigmented. All strains employed were completely inbred with the exception of ICR, which is random-bred.

A number of the strains were descended from breeding stock originally sent by other investigators, to whom I wish to express my appreciation. They are Dr. George Snell of the Jackson Laboratory (for C3H/DiSn and C3H.K), Dr. Elizabeth Russell of the Jackson Laboratory (for C57BL/6J-*bb*, C57BL/6J-W^vw, and WH-Ww), Dr. Willard Hollander of Iowa State University (for the $mi^{bw}mi^{bw}$ mutant), and Dr. Stanley Mann, then at Brown University (for *fzfzlnln*).

Results and Discussion.—Development of unitary embryos from paired eggs during the *in vitro* phase continued at a high incidence (around 95%), as previously reported.[1] Subsequent viability *in vivo* was determined for the first 4 strain combinations (Table 1), which were prepared under comparable conditions. Conditions affecting survival were not held constant among the remaining experiments. For all 4 types, 147 composite blastocysts were transferred to 15 recipients and 57 live offspring were born. Only 2 recipients had no young. Since, in the host strain, similar numbers of females from *fertile* matings yielded no progeny, failures of this frequency are to be expected. Making this correction, 57 of 128 embryos placed in all other hosts, or 45 per cent, survived to birth. (This viability at parturition exceeds all levels in published accounts of egg transfers, even when no *in vitro* experimental manipulation was involved.) One animal had head deformities and died postnatally; 6 others, though normal, were lost within a week due to maternal neglect. The remaining 50, shown in Table 1, became healthy adults, along with the others of the 109 total listed.

The experimental animals fall into two general coat color categories: those with only one color (some of which proved, with other markers, to have mosaicism in internal tissues), and those with both original colors. Despite approximately equal totals with only the dominant color (35), only the recessive color (33), or both colors (41), significant differences are evident among strain pairs and these will be analyzed on a later occasion. The presence of only one color in some individuals can be taken to reflect the action of selection, chiefly during the period of reduction in embryo size from double to normal. Even cursory examination shows that two-color animals display a great many striking features of color pattern in common. It is also noteworthy that certain kinds of color arrangements are totally absent: there are, for example, *no* individuals in which anterior and posterior halves, or



FIGS. 2–10.—Adult allophenic mice, with *standard patterns*. Both homozygous black (*CC*) and *albino* (*cc*) melanocytes are present in the C57BL/6 ↔ ICR (Figs. 2–5), C57BL/6 ↔ BALB/c (Fig. 6), and C3H/DiSn ↔ C3H. K (Figs. 7, 9, 10) individuals; *black* (*BBDD*) and *dilute brown* (*bbdd*) cells both occur in the C3Hf ↔ DBA/2 combination (Fig. 8). Note in Fig. 2 the 3 transverse head clones and 6 body clones per side (only the last one is irregular in this case). In Figs. 3–8, the same basic pattern is present with many minor irregularities, including varying degrees of invasion between neighboring clonal areas, and localized left-right asymmetries. The 8 tail clones can be seen in Figs. 9 and 10.

left and right halves, are of opposite color, nor are there any cases of pepper-and-salt intermingling. If the distribution of pigment cells were random, at least some examples of these types should be found. Clearly, then, the patterns obtained are in fact *nonrandom*. A *single*, specific kind of order is identifiable when all the two-color mice are compared.

In each of 27 animals among the total of 41, both colors are present in substantial amounts, and all animals have the same basic pattern (Figs. 2, 9, 10). Differences among them, though common, are relatively minor and appear to arise by selection (Figs. 3–8). These differences become progressively more marked among the 27 and provide a gradual transition into the remaining group of 14, in which each mouse has a large preponderance of one color and only little of the other. Despite increased variability within this second group, detailed comparisons reveal that all cases are essentially more extreme modifications, by selection, of the same basic pattern as seen in the first. The basic type, obtained most frequently, will be referred to as the *standard pattern;* it will be considered here in detail, since it serves to establish the fundamentals of melanoblast ontogeny. The 14 other cases will be collectively called *derived patterns* to indicate their status as secondary offshoots of the single *standard*. Derived patterns will be reserved for a later paper on the mechanisms of pattern modification by selection.

The occurrence of a *standard pattern* emphasizes the most conspicuous feature of these animals as well as their chief usefulness, i.e., regularity of distribution of cellular phenotypes which can be related unequivocally and causally to known allelic differences in genetic constitution. Neither the fact of discernible order nor its interpretability are conveyed by terms now in use, such as "mosaic." The word "chimera," classically implying monstrosity, seems in conflict with the normalcy of these animals and the thorough integration of their components. The mice produced by aggregating eggs will henceforth be called *allophenic* mice; that is, individuals with a simultaneous, orderly manifestation of two (or more) allelic cellular phenotypes, or *allophenes*, each with a known and distinctive genetic basis. *Allopheny* is therefore the phenomenon of concurrent display of such allelic cellular phenotypes.

The *standard pattern* is diagrammatically represented in Figure 1, and is shown in Figures 2–10. The animals are dramatically striped, with a series of broad transverse bands of alternating colors extending down the full length of head, body, and tail. Some mismatching, or differences in color intensity, occurs to some extent in every animal on either side of the dorsal midline. The sharp mid-dorsal separation indicates that the two sides are established autonomously, without physical contiguity, so that a left and a right member are actually present. Melanocytes at borders between adjacent bands are intermingled to varying degrees; admixtures may extend far into the territory of a band, but the preponderant type usually remains apparent. There are 17 successive bands down each side of an animal. The head has three per side, and the first is most often black in the $CC \leftrightarrow cc$ allophenic mice. The last head stripes end either just behind the ears or at variable distances slightly farther back. On each side, there are 6 bands on the body, and 8 on the tail. Tail bands are more difficult to resolve, as would be expected, but were distinguishable in 11 of the 27 cases (including 9 $CC \leftrightarrow cc$, 1 BBDD $\leftrightarrow$ *bbdd*, and 1 $LnLn \leftrightarrow lnln$).

The simplest explanation for these bands, consistent with all known facts of pigmentary ontogeny, is that each is a *clone* of melanoblasts, descended mitotically from a single cell. No reasonable alternative is apparent. The definitive hair-bulb melanocytes of mice do not produce their characteristic melanin product until a day after birth. But transplant experiments by Rawles[11] have demonstrated that they originate from some of the neural crest cells. The latter at first flank the longitudinal neural folds and then migrate laterally in the body wall, toward the ventrum, at 8–12 days of embryonic life. The gross direction of spread is corroborated here by the geometry of the pattern, and pigmented clones also often show gradual reduction in color toward the ventrum. Though the so-called melanoblast members of the neural crest population have remained unidentifiable microscopically, their detailed history has been rendered visible in the allophenic mice. Earlier inferences on melanoblast origins, drawn from studies of certain patterned genotypes,[12] are not supported by the evidence from the allophenics. The *standard pattern* consists of a longitudinal series of 17 presumed clones on each side, and the 34 aligned cells which initiate them can be designated *primordial melanoblasts*. Their subsequent proliferation laterally (and to some extent longitudinally, as the embryo grows) occurs as if a sheet of cells were spreading in the loose junction between epidermis and dermis. Margins of neighboring clones apparently abut against each other so that transverse movement is sustained. Barring occurrence of selection, the mode of proliferation seems similar in all clones, regardless of phenotype; no local "fields" or preferential pathways need be invoked. Each clone is a unit of perpetuated cellular individuality, and retention of identity must mean that *determination* of melanoblasts, or specific genetic function of all pigmentary loci studied here, first took place in the 34 precursor cells and then remained stabilized through numerous cell generations.

Results obtained in black and white $CC \leftrightarrow cc$ allophenics require confirmation of two kinds. First, two *positive* colors need to be observed, because of possible ambiguity in a white area (i.e., absence of pigment cells, rather than presence of amelanotic melanocytes generally typical of *cc*). Experiments were therefore conducted with $BB \leftrightarrow bb$, $BBDD \leftrightarrow bbdd$, and $LnLn \leftrightarrow lnln$ (Table 1), and the *standard pattern* was, in fact, again produced (Fig. 8). In none of these two-color animals were any white areas seen. The second line of confirmation concerns attribution of effects to individual color loci, when, in many strain combinations, other gene differences were present. For this reason, both congenic and coisogenic[13] strains were studied. The C3H/DiSn and C3H.K strains are a congenic pair known to differ at only two loci (*CC* and $H\text{-}1^aH\text{-}1^a$, as compared with *cc* and $H\text{-}1^bH\text{-}1^b$). These yielded allophenics with the *standard pattern*. Differences at the *H-1*-locus cannot be implicated since the C3Hf and DBA/2 strains are both $H\text{-}1^aH\text{-}1^a$, yet that combination led to the same pattern (Table 1). Nevertheless, congenic strains are obtained by repeated backcrossing and may conceivably retain undetected, closely linked genic differences. Therefore, final critical proof was sought and secured with the C57BL/6J $\leftrightarrow$ C57BL/6J-*bb* coisogenic pair; these are identical except for the $BB \leftrightarrow bb$ differential, since *b* arose as a mutation in the C57BL/6J inbred strain. In addition, the presence of two positive colors in the same pattern satisfies the first requirement discussed above. We can conclude that *different*

allelic phenotypes at only a single locus in primordial melanoblasts are a sufficient condition for production of a specifically ordered pattern.

Summary.—Many adult mice with two separate genetic types of cells have been obtained from experimentally aggregated pairs of genotypically different cleavage-stage embryos. These mice are called *allophenic*, since they display two allelic cellular phenotypes with a known genetic basis for each. Experiments in which cells differing in melanocyte genotype were combined suggest the probable developmental foundation for coat-color differentiation. Color distribution in the allophenics occurs as if all melanocytes in the adult coat are *clonally* derived from 34 *primordial melanoblasts* arranged in two longitudinal mid-dorsal chains of 17 each, in which melanocyte-determining genes first become active in the early embryo, and remain stabilized. If two allelic cellular phenotypes are present (for several independent loci tested, and presumably for all other pigmentary loci normally acting within melanoblasts), the initial melanoblast types seem first to take up alternating rather than random positions in the chains. Each cell appears to proliferate laterally, and to a lesser extent longitudinally, to fill the available space between epidermis and dermis, bounded on each side by neighboring clones. This mode of proliferation is evidently similar in all clones, unless gene-specific cell selection supervenes. The unselected pattern, or *standard pattern*, consists of broad transverse bands of alternating color. This complete clonal history, the first available for a vertebrate cell type, provides the basis for analyzing known melanocyte patterns in mice, whether due to autosomal or sex-linked genes, and for elucidating the genetic mechanisms which control orderly pattern differentiation.

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